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**TITLE:** Cotargeting VEGF and Neuropilins With Bevacizumab and Secreted Wnt Inhibitors in Prostate Cancer

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14. ABSTRACT The objectives of this proposal are to test hypotheses: 1) WIF1 IgG fusion protein has an additive or synergistic effect with anti-VEGF therapy to inhibit tumor growth and metastasis; and 2) NRP2 is a wnt target gene and predicts prostate cancer progression. The WIF1 IgG fusion protein expression construct was re-engineered with pFUSE-hlgG1-Fc vector, which was suggested to have good stability in plasma and good pharmacokinetic properties. However, the produced WIF1-Fc fusion protein was still cleaved into an about 35 kd fragment. Bioinformatics analysis suggested that the potential cleavage sites are Lys339 and Arg340. Mutagenesis assays are in progress to solve the problem of the WIF1-Fc fusion protein cleavage. In addition, our results showed that Bevacizumab increased Wnt signaling and expression of c-Met and NRP2 protein, leading to increased migration and invasion of PC3 cells. Ectopic expression of WIF1 and stable knock-down of NRP2 expression in PC3/LN4 cells decreased cell migration, invasion and tumor growth at the prostate of SCID mice and lymph node metastasis. NRP2 protein levels were elevated in castration-resistant prostate cancer tissues and correlate to LEF1					
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## Introduction

Anti-Vascular Endothelial Factor (VEGF) based anti-angiogenesis therapy is becoming one of major approaches for treatment of several advanced and metastatic cancers, including castration-resistant prostate cancer (CRPC) [1]. However, development of resistance to anti-VEGF therapy, in particular to Bevacizumab treatment, appears to be very common and inevitable [2]. Currently the underlying mechanisms for Bevacizumab resistance are largely unknown.

Neuropilin-2 (NRP2) and c-Met are co-receptors with each other and with VEGF receptors [3, 4]. Accumulating studies have implicated that both NRP2 and c-Met play important roles in tumor progression and metastasis and are involved in angiogenesis [3, 4]. Based on our preliminary bioinformatics analysis and experiments, we hypothesize that Bevacizumab treatment results in up-regulation of NRP2 and c-Met expression, leading to resistance to Bevacizumab and tumor progression. C-Met is a known Wnt target gene [5]. We also hypothesize that NRP2 is a Wnt target gene, and that expression of c-Met and NRP2 can be down-regulated by a secreted Wnt inhibitor [i.e. Wnt inhibitory factor-1 (WIF-1)]. Therefore, it is possible that WIF-1, in combination with Bevacizumab, can improve the efficacy of Bevacizumab therapy in CRPC and overcome the resistance to Bevacizumab.

To test these hypotheses, three specific aims are proposed as follows: Aim 1: To determine whether WIF1 recombinant protein has an additive or synergistic effect with anti-VEGF therapy to inhibit tumor growth and metastasis in clinically relevant orthotopic models. Aim 2: To determine whether NRP2 is a transcriptional target of canonical Wnt/TCF signaling and contribute to the combined effect of WIF1 and Bevacizumab on cell proliferation, migration and invasion. Aim 3: To determine whether the expression of NRP2 alone or in combination with VEGF receptors or c-Met will be associated with MVD and WIF1 expression, and will be predictive for clinical and biochemical progression-free survivals of PCa patients, as well as for development of metastasis.

The first stage of this proposal is 1) to determine whether NRP2 is transcriptionally regulated by the Wnt pathway, and 2) to produce a recombinant WIF-1 protein (i.e. WIF-1 human IgG fusion protein) that can be used as a therapeutic agent for inhibiting Wnt signaling in prostate tumors in animal models and for combined therapy with Bevacizumab.

The second stage of this proposal is 3) to determine the efficacy of WIF-hIgG alone or in combination with Bevacizumab for treatment of CRPC in xenograft mouse models, and 4) to determine whether NRP2 is at least in part required for WIF-hIgG and Bevacizumab mediated inhibition of cell growth, migration and invasion.

The third stage of this proposal is 5) to determine whether NRP2 alone or in combination with VEGF receptors or c-Met is an independent predictor for prostate cancer biochemical recurrence or metastasis.

During the past years, we are still stuck on the first stage of this proposal and unable to produce WIF1-Fc fusion protein with good stability. However, we are close to solve this problem by identifying potential cleavage sites, which are Lys339 and Arg340. Mutagenesis assays are in progress to solve the problem of the WIF1-Fc fusion protein cleavage.

We also have made significant progress in this research project by elucidating mechanisms of bevacizumab resistance in prostate cancer and functional role of NRP2 and WIF1 in prostate cancer cell migration, invasion, tumor growth and metastasis. We described our negative and positive findings according to our Statement of Work as follows.

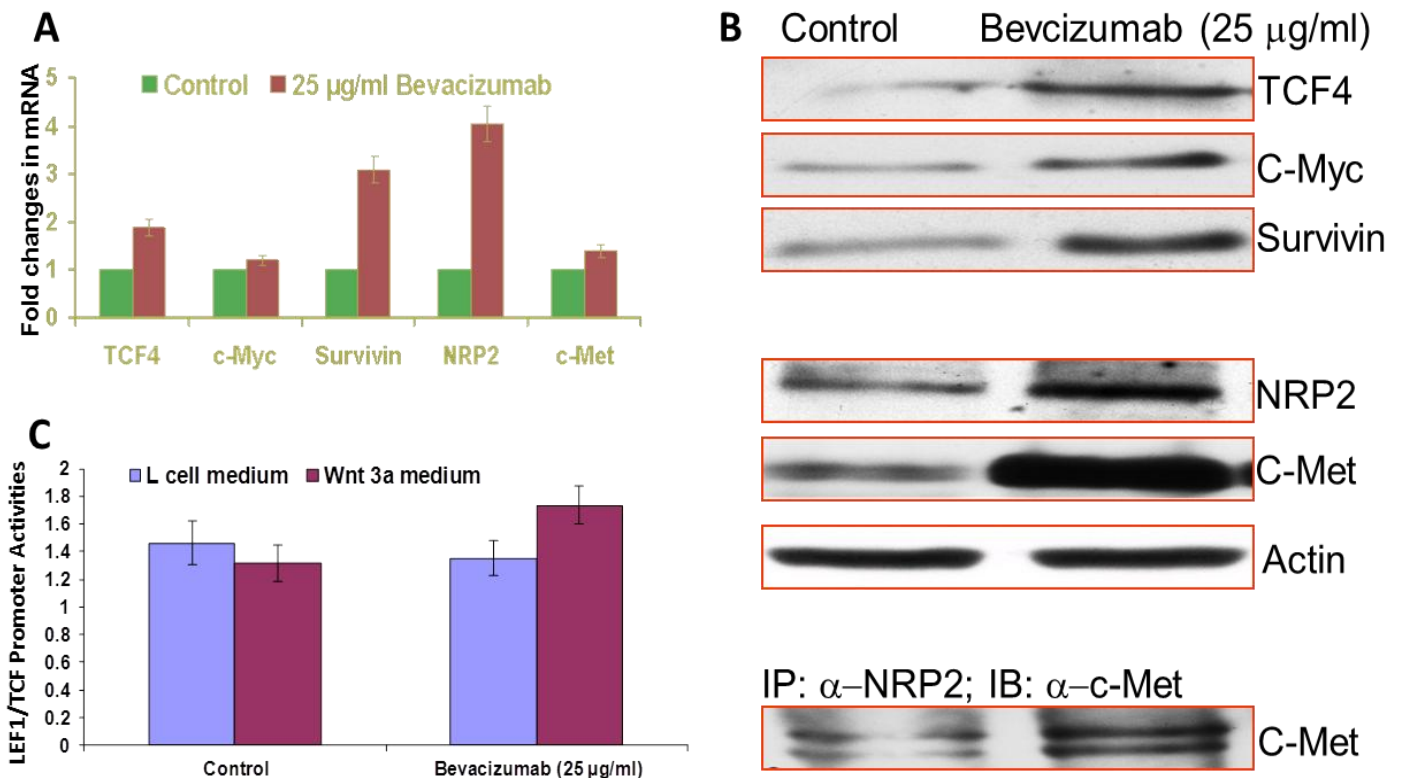
## BODY:

**Task 1. To determine whether WIF1 recombinant protein has an additive or synergistic effect with anti-VEGF therapy to inhibit tumor growth and metastasis in a clinically relevant orthotopic model.**

- (1) Animal protocol was approved by the UCI IACUC (protocol number 2011-2938).
- (2) The produced WIF1-Fc fusion protein was still cleaved into an about 35 kd fragment. Bioinformatics analysis suggested that the potential cleavage sites are Lys339 and Arg340.

**Task 2. To determine whether NRP2 is a transcriptional target of canonical Wnt/TCF signaling and contribute to the combined effect of WIF1 and bevacizumab on cell proliferation, migration and invasion.**

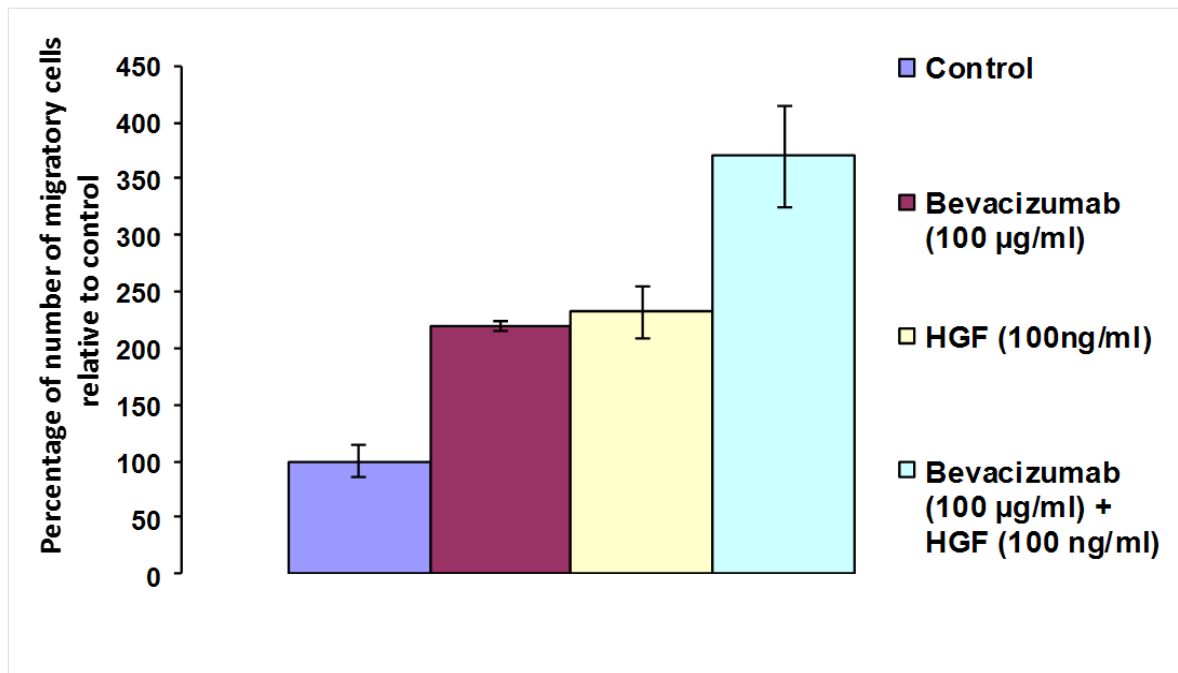
- (1) Bevacizumab treatment of PC3 cells resulted in an increased expression of TCF4, known Wnt target genes (c-Myc and survivin) and NRP2 and c-Met, and Bevacizumab treatment increased the responsiveness of PC3 cells to Wnt 3a stimulation. Using real-time RT-PCR, **Figure 1A&B** show that Bevacizumab treatment at a concentration of 25  $\mu\text{g/ml}$  for 24 hours increased the mRNA and protein expression of TCF4, c-Myc, survivin, NRP2 and c-Met. Immunoprecipitation also revealed that Bevacizumab enhanced the binding of NRP2 to c-Met (**Figure 1 B the bottom panel**). PC3 cells were treated with vehicle control (PBS) or 25  $\mu\text{g/ml}$  Bevacizumab. 24 hours later, these cells were transfected with TOPFLASH luciferase constructs and control vector and treated with Wnt 3a conditioned medium or L cells control medium. **Figure 1C** shows that Bevacizumab treatment significantly increase the responsiveness of PC3 cells to Wnt 3a stimulation compared to control treatment ( $P<0.05$ ).



**Figure 1** The effect of bevacizumab treatment on the Wnt signaling and expression of its target gene and NRP2 and c-Met in PC3 cells.

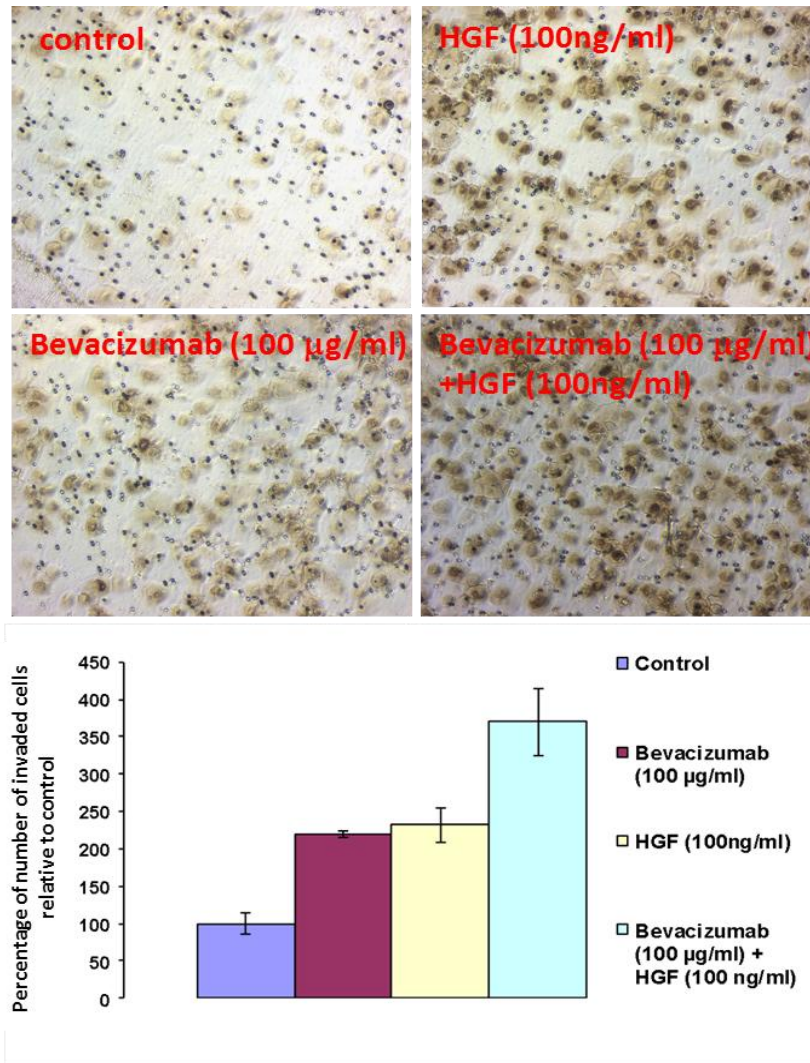
- (2) Bevacizumab treatment of PC3 cells increased its migration and invasion, as well as HGF-induced cell migration and invasion.

PC3 cells were treated with vehicle control (PBS), 100  $\mu\text{g/ml}$  bevacizumab alone, 100 ng/ml Hepatocyte Growth Factor (HGF), and Bevacizumab plus HGF for 24 hours. Cell motility was measured by average number of cells migrating through a control, uncoated insert. Number of cells on each membrane was averaged from 10 fields ( $\times 100$ ). **Figure 2** shows that both bevacizumab and HGF treatment significantly increased the migration of PC3 cells compared to control treatment, and that Bevacizumab and HGF combination further enhanced the migration of PC3 cells compared to the treatment alone (**Figure 2**). This result suggested that Bevacizumab induced cell migration may be involved in the HGF mediated signaling pathway.



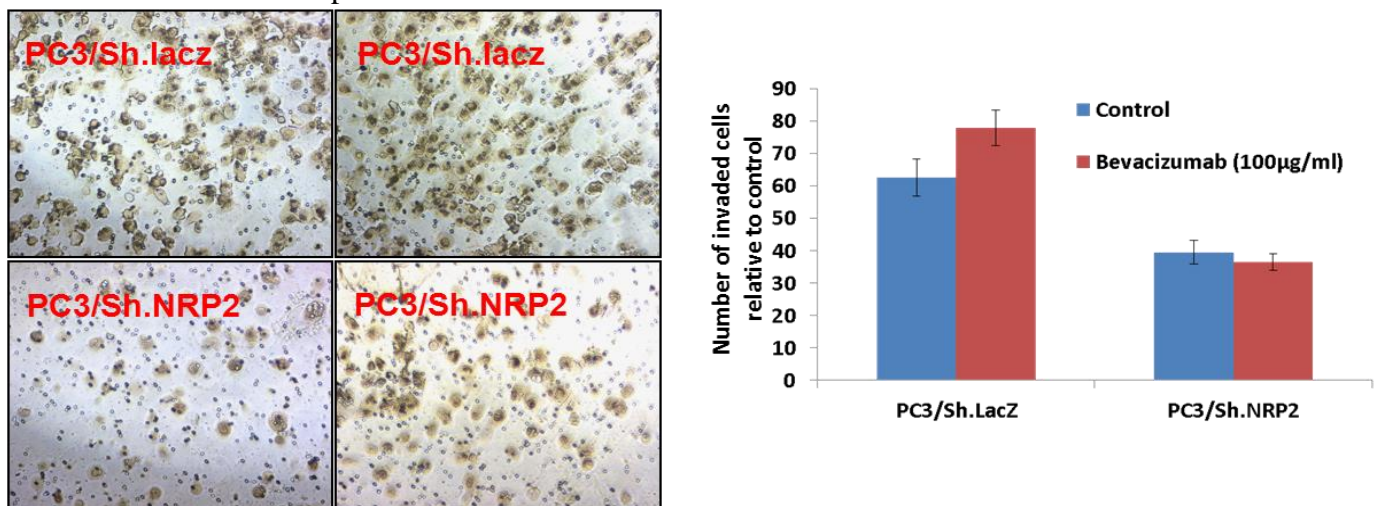
**Figure 2** The effect of Bevacizumab or HGF alone or their combination on the migration of PC3 cells.

Using Matrigel invasion assay. The capacity of PC3 cells to invade through a Matrigel-coated membrane under the same treatments as described above was measured and expressed as average number of migrated cells on the lower surfaces of triplicate membranes and adjusted by control group. Bevacizumab-, HGF, and their combination treated cells exhibited a significant increase in invasive capacity (by 120%, 34%, and 260%, respectively) compared with control cells (Student's t test,  $P < 0.05$  to  $P < 0.01$ , respectively; **Fig. 3**).



**Figure 3.** The effect of Bevacizumab or HGF alone or their combination on the invasion of PC3 cells.

(3) Knock-down of NRP2 expression attenuated the Bevacizumab induced invasion of PC3 cells



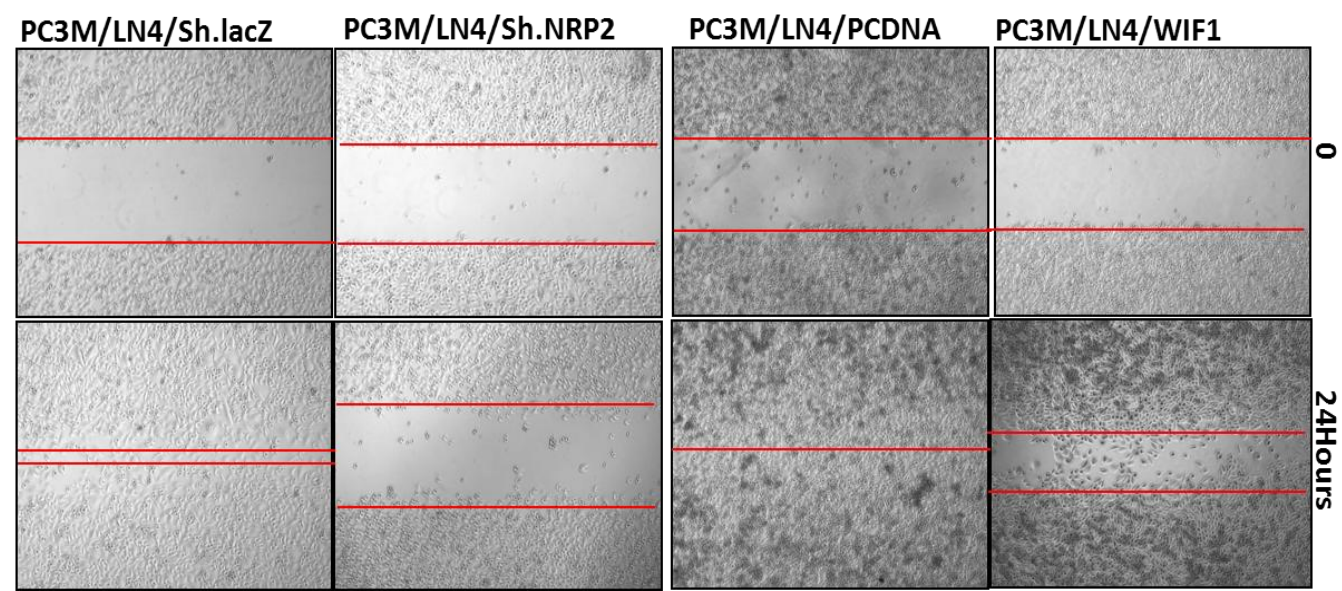
**Figure 4.** Suppression of NRP2 expression by ShRNA reduced Bevacizumab induced cell migration.

PC3 cells were stably transfected with vector control or NRP2 shRNA to knock-down the expression of

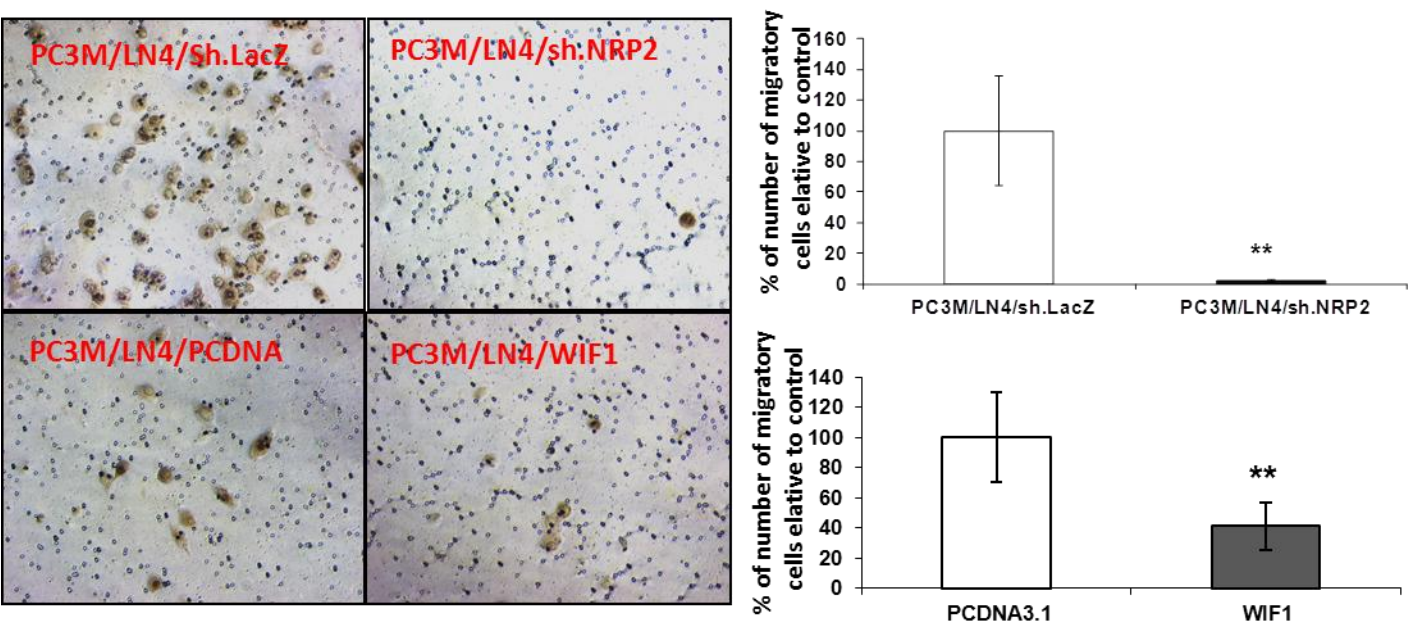


NRP2 in PC3 cells. **Figure 4** shows that Bevacizumab increased the invasiveness of PC3 cell stably transfected with Sh LacZ but not that PC3 cells with stable suppression of NRP2 by ShRNA.

(4) WIF1 overexpression and NRP2 knock-down reduced migratory capacity and invasiveness of PC3M/LN4 cells.



**Figure 5.** The effect of WIF1 and NRP2 expression on cell migration .

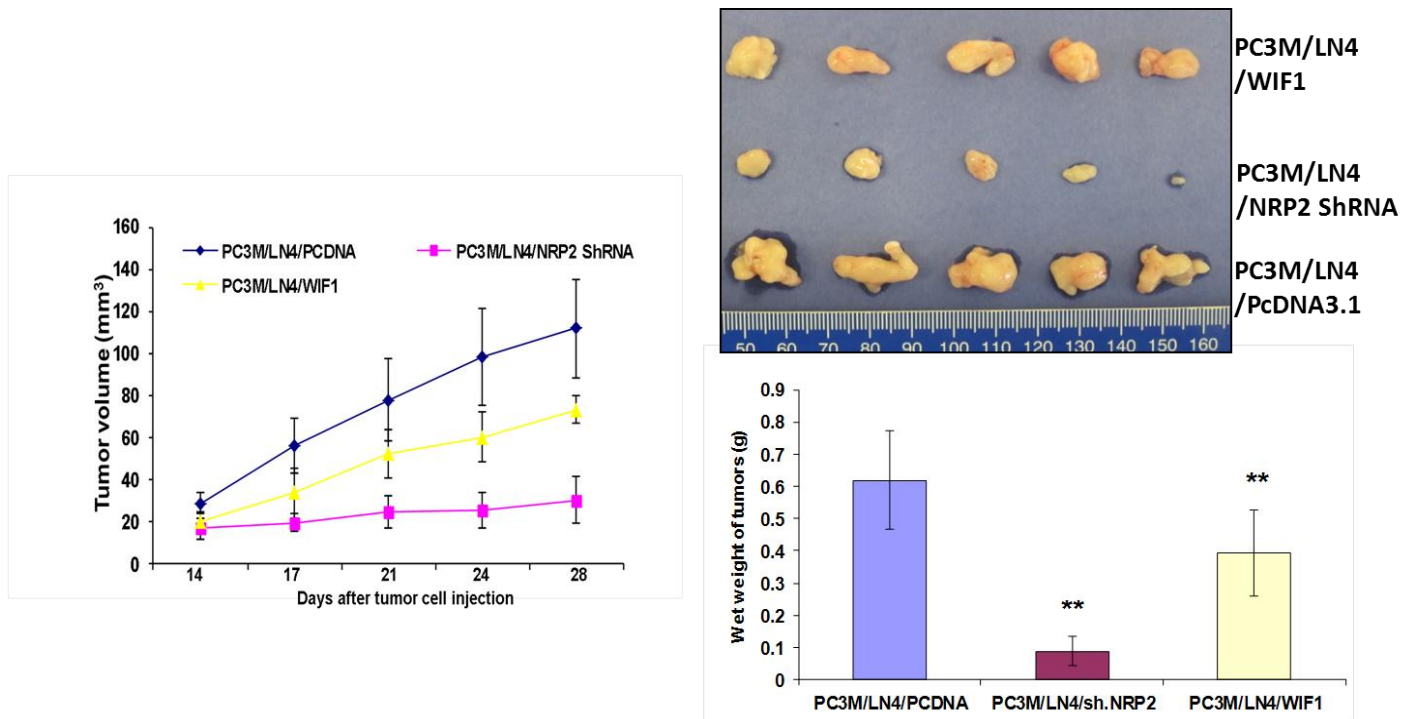


**Figure 6.** The effect of WIF1 and NRP2 expression on cell invasion.

PC3M/LN4 cell line is a variant of PC3 cells that were derived from lymph node metastasis. **Figure 5 and 6** show that both WIF1 overexpression and knock-down of NRP2 expression resulted in a slow migration and invasion of PC3M/LN4 cells.

(5) WIF1 overexpression and NRP2 knock-down decreased tumor growth in xenograft models in nude mice

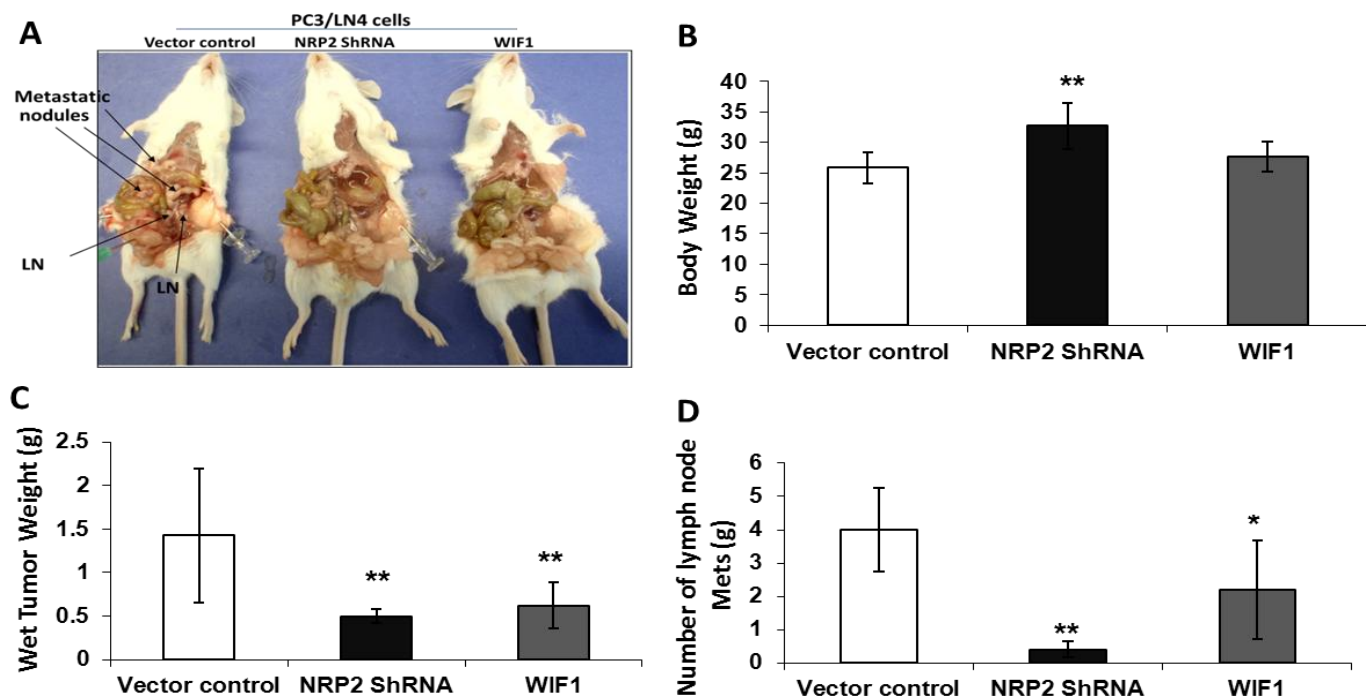




**Figure 7.** The effect of WIF1 and NRP2 expression on tumor growth in a xenograft model in nude mice.

$1 \times 10^6$  PC-3M/LN4 cells that were transfected with vector control, WIF1, or NRP2 shRNA were injected into the left flank of nude mice. Parental PC-3M/LN4 cells and vector control transfectants exhibited more rapid tumor growth over time compared to WIF1 or NRP2 ShRNA transfectants (**Figure 7**, ANOVA test,  $P < 0.01$ ). Knock-down of NRP2 is more effective in reduction of tumor growth.

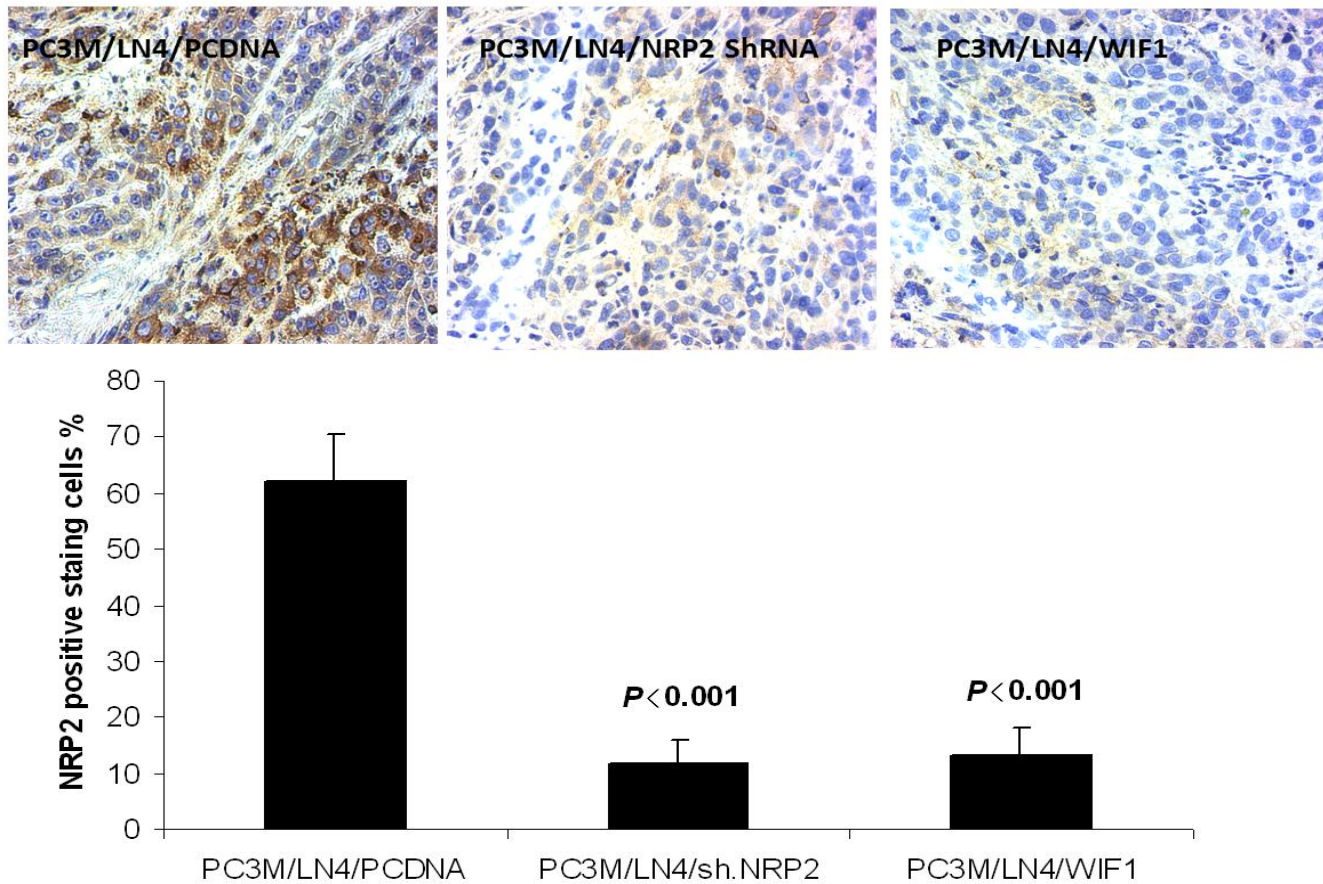
**(5) WIF1 overexpression and NRP2 knock-down decreased tumor growth and metastasis in an orthotopic models in SCID mice.**



**Figure 8.** The effect of WIF1 and NRP2 expression on tumor growth and metastasis in an orthotopic model in SCID mice.

**Figure 8 shows that** PC3M/LN4 cells with WIF1 overexpression or NRP2 knockdown exhibited lower weight of tumor bearing prostate and less number of lymph nodes with prostate cancer in SCID mice compared to those transfected with vector control (ANOVA test,  $P<0.05$  to  $P<0.01$ ).

Immunohistochemistry staining of tumor tissues revealed that WIF1 overexpression and NRP2 knock-down also reduced the expression of NRP2 in vivo in tumor tissues (**Figure 9**)



**Figure 9.** The effect of WIF1 expression and NRP2 knock down on NRP2 expression in tumor tissues.

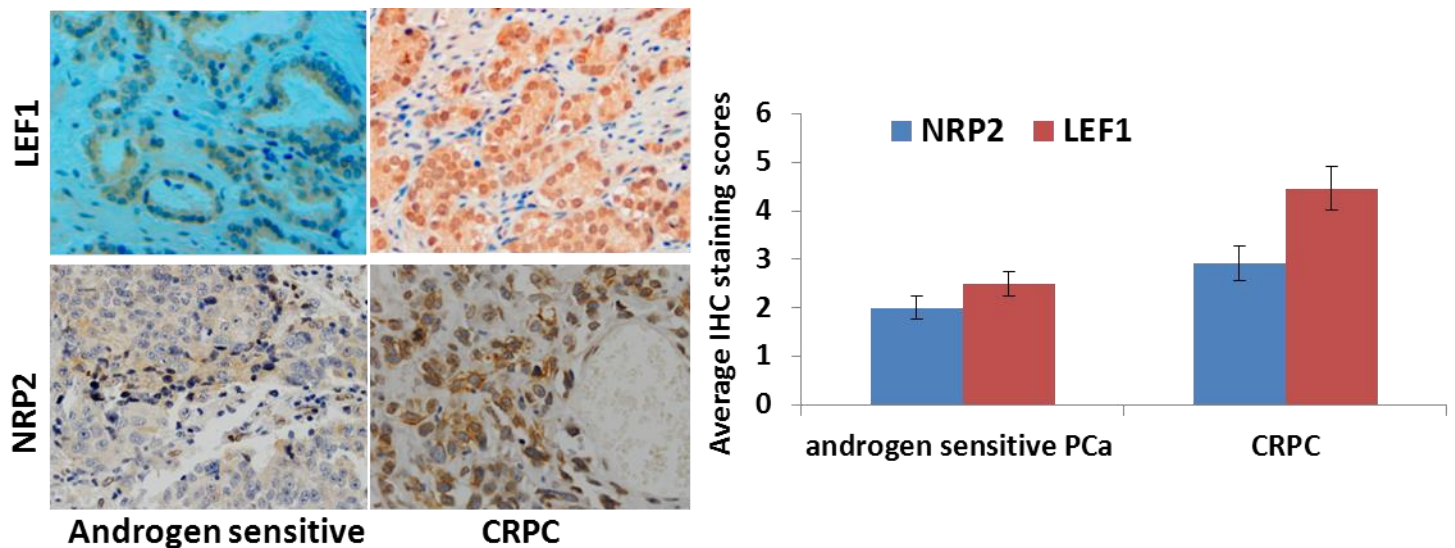
**Task 3. To determine whether the expression of NRP2 alone or in combination with VEGF receptors or c-Met will be associated with lymphatic vessel density, MVD, and WIF1 expression, and will be predictive for clinical and biochemical progression-free survivals of PCa patents, as well as for development of metastasis.**

- (1) Both LEF1 and NRP2 are overexpressed in Castration-resistant prostate cancer tumor tissues and their expression levels are positively correlated.

A CRPC Tissue Microarray (TMA) that consists of 40 CRPC cores (20 cases) from palliative transurethral resection in men with CRPC was constructed by Dr. Jiaoti Huang, Professor of Pathology at University of California, Los Angeles. We have obtained this TMA for the following study. Additional 40 cases are also available to us from collaboration with Dr. Jiaoti Huang. The final staining score (1-2, negative; 3 to 4 weak positive and 5 to 6 strong positive) was calculated as the sum of the percent staining multiplied by the

corresponding intensity level. The average IHC staining scores of LEF1 and NRP2 are higher in CRPC tissues than those in androgen sensitive prostate cancer tissues from radical prostatectomy.

We have also observed very similar expression patterns between NRP2 and LEF1 in these PCa tissues. The staining scores of NRP2 and LEF1 are highly and positively correlated (correlation coefficient  $\gamma = 0.2982$ ).



**Figure 10.** The expression of NRP2 and LEF1 in androgen sensitive PCa and CRPC.

### **Key Research Accomplishments.**

1. Identification of two potential cleavage sites (i.e. Lys339 and Arg340) of the WIF1-Fc fusion protein.
2. Bevacizumab increased Wnt signaling and expression of NRP2 and c-Met in PC3 cells.
3. Bevacizumab treatment resulted in an enhanced cell migration and invasion of PC3 cells and knock-down of NRP2 attenuated these effects of Bevacizumab.
4. WIF1 re-expression and NRP2 knock-down reduced cell migratory capacity and invasiveness.
5. WIF1 re-expression and NRP2 knock-down reduced tumor growth in a xenograft model in nude mice.
6. WIF1 re-expression and NRP2 knock-down decreased tumor growth at the prostate of SCID mice and lymph node metastasis in an orthotopic model of prostate cancer in SCID mice in human specimens.

### **Reportable Outcomes**

1. Bevacizumab increased the Wnt signaling, which may lead to enhance tumor cell migration and invasion through NRP2 up-regulation.
2. NRP2 plays an important role in prostate cancer tumorigenesis and metastasis and is a potential target for treatment and prevention of prostate cancer recurrence.
3. WIF1 is a tumor suppressor in prostate cancer and can be used as a therapeutic agent for treatment of human prostate cancer.

### **4. Published Abstract.**

Noriko N. Yokoyama, Zheng Sun, Toshinori Sakai, Bang H. Hoang, **Xiaolin Zi**. Robo1, a new target, by Wnt and androgen receptor signaling in castration resistant prostate cancer. In: Proceedings of the American

### **Conclusion:**

Bevacizumab resistance is common in its treated cancers. In this study, we provide evidence that Bevacizumab can increase Wnt signaling, leading to enhanced migratory capacity and invasiveness of prostate cancer cells. In addition, we have shown that NRP2 expression plays an important role in prostate cancer tumorigenesis and metastasis. Our results also suggested that WIF1 could be a potential therapeutic agent for combination therapy with Bevacizumab.

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